Investigating Photonic nanostructures for reproducible characterization of bacterial spores

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ABSTRACT

Raman spectroscopy has proven to be a plausible solution to the difficult challenge of on-site detection of biological threats. Adding to the challenge is the fact that many biological species, spores specifically, have relatively low scattering cross sections. The intrinsic need to detect these threats at low concentrations and in the presence of strong background signals necessitates the need for surface enhancement schemes. With an available technique to quickly identify bacterial spores, we are investigating the existence of spectral differences between target species in order to incorporate library technologies with the on-site sensor. We are examining many of the reported substrate classes such as: nano-sphere lithography (NSL), Film over nano-sphere (FONS), nano-shells, electrochemically roughened metals, and dispersed and immobilized colloids. Key aspects of this work include discerning what architectural features provide the largest enhancement and reproducibility. We will present preliminary results of bacterial spore identification as well as a comparison of the substrates studied.
Keywords: Surface-enhanced Raman Spectroscopy, bacteria, biological detection

1. INTRODUCTION

The detection of biological threats, specifically spores, presents a challenging problem to the scientific community. Obviously, a detection method is necessary that can detect multiple threats in real time and allow a reaction that is much faster than the onset of fatal symptoms. According to a recent report, the progression from symptoms to death related to Anthrax exposure can be as quick as three days. [(1)] DNA hybridization is a common technique addressing this problem. However, DNA hybridization and its many variations require *a priori* knowledge of the targets, are specific to only one threat per sensor or sensor element, and are susceptible to false alarms. Therefore we propose a surface enhanced Raman spectroscopy (SERS) approach that might detect biological threats at trace levels on-sit, and in the presence of strong and possibly interfering backgrounds.

Difference vibrational spectroscopy has a proven track record solving this class of problems [(2)] as the vibrational energy levels of a molecule or assembly of molecules is quantitatively distinct. Information rich techniques such as Raman, and FTIR spectroscopy have both been used to identify bacterial species [(3, 4)], but it is generally accepted that Raman has greater potential in real world environments due the strong absorbance of water in FTIR measurements. The recent advances in technology such as diode lasers, the holographic notch filter, and compact spectrographs with sensitive CCD cameras have also added to the more robust ability of Raman spectroscopy. [(5)] However, many biological species, bacterial endospores specifically, have relatively low scattering cross sections. The intrinsic need to detect these threats quickly and in their natural/complex environments necessitates the need for surface enhancement schemes. Indeed, over 25 years of research have been conducted on the phenomenon of surface enhancement. [(6)]

SERS was first reported of pyridine over roughened silver electrodes. [(7)] The SERS effect has since been determined to be the result of a magnified electric field at the surface of nanometer scale features on a noble metal's surface. The electric field is enhanced only in the acute region of the features' surfaces (*i.e.* <100 nm) and only for select wavelengths that match the plasmon resonance of the overall surface. In addition the orientation of the molecule

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 OCT 2005	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Investigating Photonic nanostructures for reproducible characterization of bacterial spores	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **U.S. Army Research Laboratory, Sensors and Electron Devices Directorate 2800 Powder Mill Road, Adelphi, MD 20783**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM001851, Proceedings of the 2003 Joint Service Scientific Conference on Chemical & Biological Defense Research, 17-20 November 2003. , The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**11**	

on the surface of the metal can also add enhancement through charge transfer. What has become apparent is that the specific architecture of the surface, *i.e.* the spacing, size, composition, and thickness of the nanoscale features plays an important role in the amount of enhancement and the wavelength of plasmon resonance. Recent studies have modeled the expected localized surface plasmon resonance for NSL and nanoshell substrates [8, 9], and have also shown the expected electric field intensity near the surface of single particles that make up the arrayed substrate [10, 11]. However, the total electric field above an array substrate has not been modeled, and the expected enhancement of molecules or particles much larger than the features have not been approached in depth. What holds promise are substrates that are "tunable" in various fashions with respect to the above surface architecture, as this will provide insight on large particle SERS while conserving the localized surface plasmon resonance matching the preferred excitation source.

Colloids produced via the reduction of Au and Ag salts and electrochemically roughened electrodes are used regularly to achieve SERS spectra. We have reported an enhancement factor of 24 and a limit of detection of 1 x 10^4 cfu/mL for *B. subtillis* and *E.coli*. [12] While the general size of the colloids in a sol can be controlled and thus matched to certain available wavelengths, the specific morphology of the surface of a colloid is not reproducible. Furthermore, the target analyte may induce aggregation of the colloid. This leads to broad variations in intensity/enhancement based on which site along the colloid is illuminated or complexed with the target analyte. In order to form a uniform surface architecture and therefore, reproducible characterization of weakly scattering analytes, novel fabrication techniques have been developed. In addition, repulsive effects due to the negative surface charge of both bacterial spores and colloid particles may prevent the spores from approaching closely enough to the colloid surface to experience the strongest part of the enhances electric field.

We have previously reported on FONS, and Au-island films. [13] We have expanded this comparison qualitatively to nano-shells and electrodelessly plated FONS substrates in a comparative fashion with respect to fabrication and SERS enhancement. The results follow.

2. EXPERIMENTAL

2.1. Spectrometers

Two spectrographs have been used in this work. The system employed at the United States Military Academy consists of a J-Y TRIAX 550 spectrograph (focal length .55m with a 1200 grooves/mm grating) and the 647.1 line of a Coherent 300C Kr-ion laser. The laser is coupled to samples and the spectrograph via an In-photonics Raman probe. The probe has a nominal focal length of 5 mm and collects the Raman signal in the 180^o geometry to be carried back to the entrance slit of the spectrograph. Rayleigh scatter from the fiber is rejected via an additional Kaiser SuperNotch ^{TM} holographic filter placed before the entrance slit. A thermoelectrically cooled 1024 × 256 pixel back illuminated CCD Spetrum-1 camera that was thermoelectrically cooled to -44 °C was used to record spectra. This system was used to obtain the data for figures 6 & 7.

The system used at ARL is composed of a Coherent Innova 70 mixed gas Kr-Ar ion laser (also @ 647.1 nm). The spectrograph is an Acton SpetraPro 275. This spectrograph, like the TRIAX, is also equipped with three gratings. All spectra in this report, using this instrument, were acquired with the 1800 grooves/mm grating, and spectra were recorded with a Princeton Instruments 536 × 388 pixel Intensified CCD camera which is water cooled to -30 ^{o}C. Coupling between the laser, sample and spectrograph is also accomplished with another In-photonics 647.1 Raman fiber optic probe. The remaining data was acquired at the U.S. Army Research Laboratory.

2.2 Substrate Preparation

FONS substrates were prepared by one of three methods. The first method involves drop coating 400 nm carboxylated polystyrene latex beads (Polysciences, Inc.) onto a clean (soaked in piranha solution @ 60 ^{o}C for 10 minutes and rinsed with deionized water) glass coverslip. The coverslip was then gently rolled by hand to evenly distribute the spheres over the entire surface until dry. Au was vacuum deposited onto the latex sphere mask to a nominal thickness of 200 nm. The other two methods utilize an electrodeless plating method of Au or Ag. The first of these substrates uses 1.0 μm carboxylated polystyrene latex beads (Polysciences, Inc.) drop coated onto a clean (rinsed in methanol and dried) glass coverslip, and dried in an oven at approximately 125 ^{o}C. A new razor blade is then used to separate the latex sphere mask from the glass slide. This provides a relatively large well ordered array of the latex

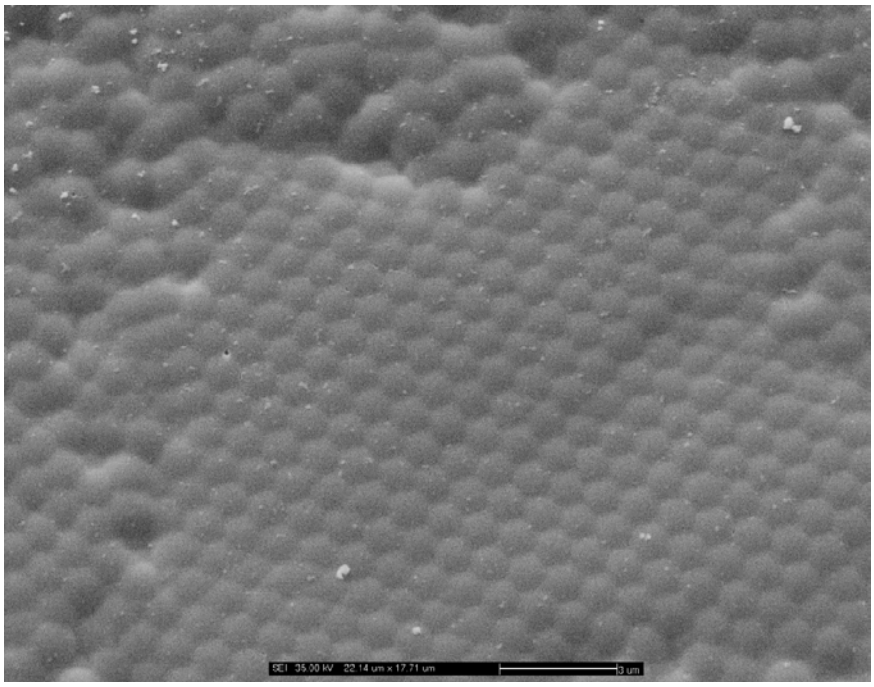

Figure 1: SEM image of bottom surface of a carboxylated latex sphere mask produced by thermal evaporation on glass coverslip; scale bar is 3 µm

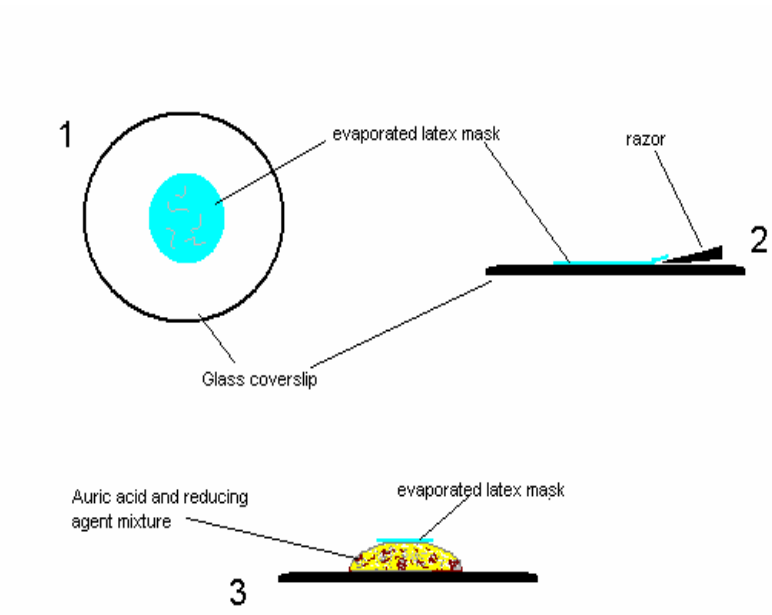

Figure 2: Steps one through 3 depict the creation of the wet FONS using 1.0 µm carboxylated latex spheres: step 1) the solution of spheres is evaporated in a convection oven at 125 ^{o}C 2) a razor is used to separate the mask from the glass coverslip and expose the bottom ordered face of the mask 3) the latex mask floats on top of a solution of auric acid and the reducing agent

spheres on the bottom side of the masks, as can be seen in figure 1. The bottom surface of the substrate floats on top of, and is exposed to, the Au solution that will be reduced. Gold [2mM $AuHCl_4$] is immobilized on the mask by citrate [25mM] reduction in the presence of sodium hypophosphite [70mM] for two hours in a volume ratio of 1:0.2:1 respectively. This process is depicted in figure 2. After the initial reduction, the substrate is rinsed with DDI water, which leaves behind gold immobilized on the surface of the carboxylated spheres. Subsequent deposition of gold [20mM $AuHCl_4$] onto the mask proceeded via reduction with formaldehyde in a 1:1 ratio, and lasted up to 72 hours. Substrates were then rinsed to quench the reaction, flipped over and allowed to dry in air before SERS spectra were collected. The third type of FONS substrates were produced with 190 nm diameter aminated polystyrene latex spheres (Bangs Labs, Fishers, IN). These substrates were produced via a small computer fan used as a make-shift spin coater. Glass coverslips were polished with a "chemwipe" and placed on the fan without further cleaning. The fan was allowed to reach its maximum speed at which time three drops of the aminated sphere solution were placed on the rotating glass coverslip. The substrates continued to spin until dry and then removed from the fan. A solution of 15 nm gold colloid is then placed over the entire substrate and allowed to sit overnight, covered, in a Petri dish. The excess colloid is washed away with DDI water and subsequent deposition of gold can proceed by reduction of auric acid by formaldehyde. SER spectra of *bacillus stearothermophilus* American type culture collection **(ATCC)** 12980 (Raven Laboratories) were acquired by modifying the above aminated sphere substrate, by

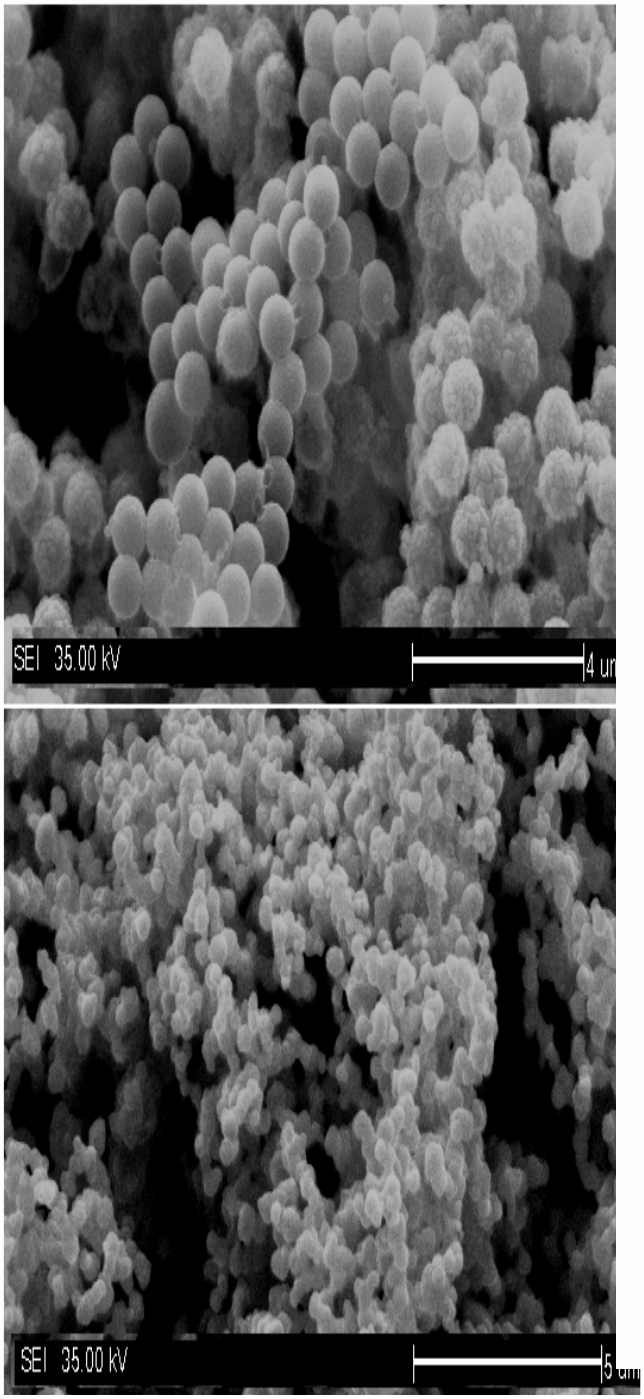

Figure 3: SEM image of nanoshell film, a) using 860 nm core latex shells. Evident are islands on the beads versus a smooth continous shell, as well as beads that have little Ag immobilized; b) shows the same type of film produced by the reaction with no latex spheres present

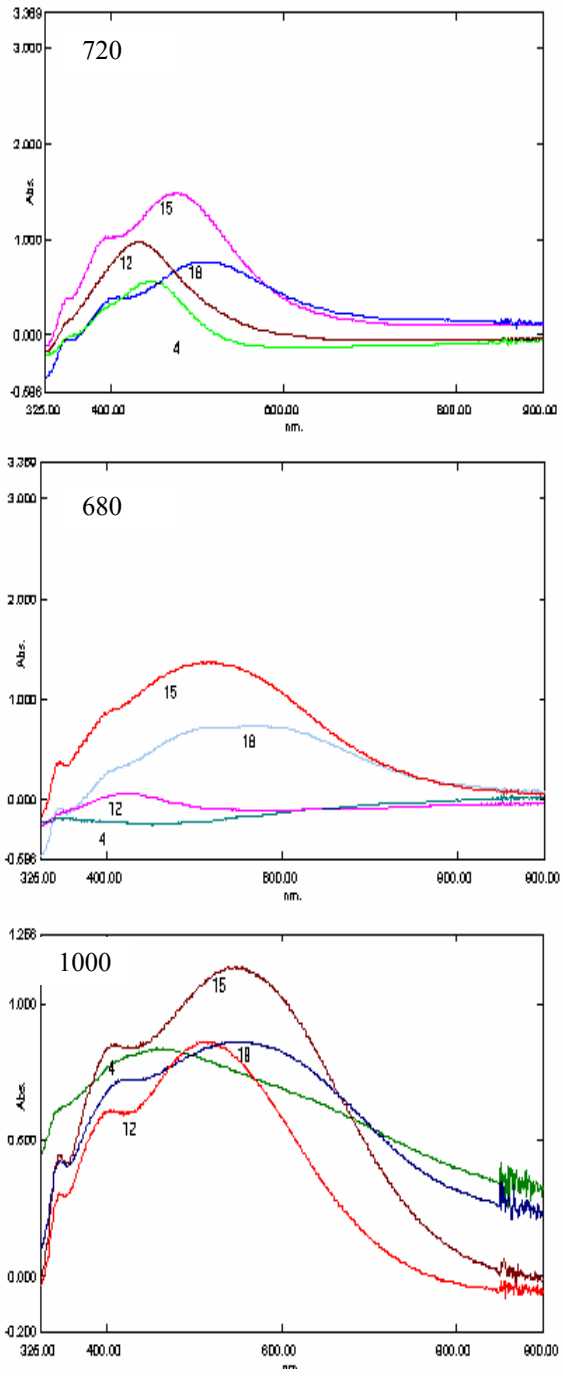

Figure 4: UV-vis extinction spectra of Ag nano-shells using different size carboxylated latex sphere cores; the numbers in the upper left hand corner indicate the diameter of the latex bead used as the core, while the numbers under each curve represent the amount of AgNO3 added to the reaction in µL x 100

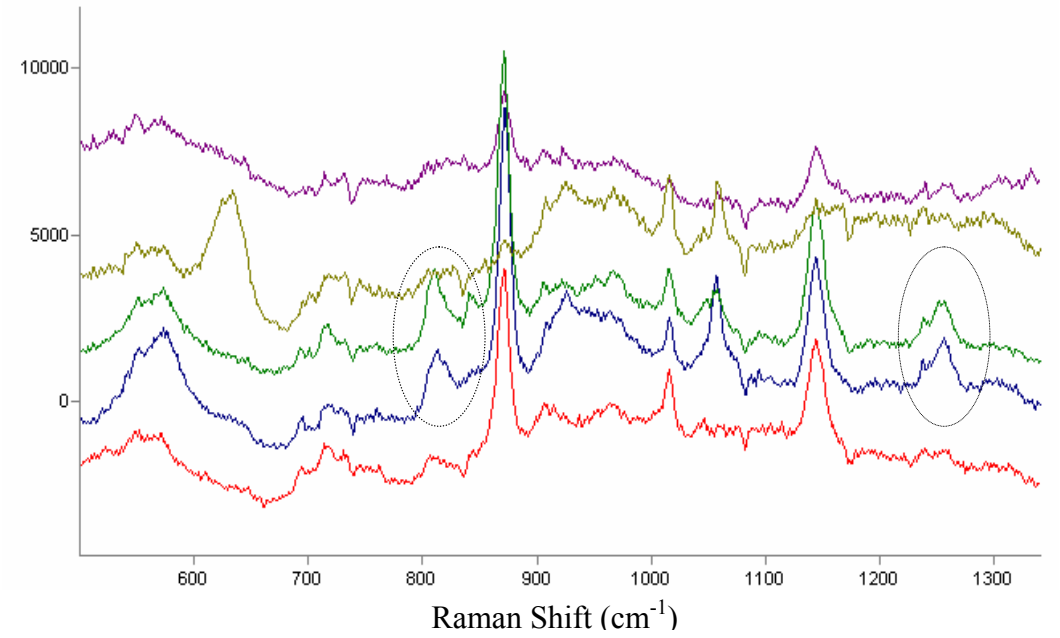

Figure 5: Spectra are offset for clarity, from top to bottom: SER spectrum of *bacillus megaterium* after substrate was allowed to dry for 12 hours; SER spectrum of *bacillus megaterium* over different spot on substrate 24 hours later; SER spectrum of *bacillus stearothermophilus* after initial 12 hours of drying, SER spectrum of *bacillus stearothermophilus* over different spot on substrate 24 hours later; bottom trace is characteristic spectrum of the substrate alone. The dashed circles highlight areas where the *bacillus stearothermophilus* show features absent in *bacillus megaterium* and the substrate alone

first applying 50µL aliquot of a 1 x 10^6 cfu/mL suspension to the bare aminated sphere mask. Gold was deposited as described above, first with 15 nm colloid, then reduction of auric acid by formaldehyde. Prior to interrogation, silver [7 mM AgNO3] was deposited onto the substrate by reduction with formaldehyde under basic conditions. For clarity these substrates will be referred to as vacuum FONS, wet FONS, and amine-wet FONS respectively as they are described above.

Au-island films were provided to us by Zyvex, Inc. and used without further treatment. Metal island films can be produced by vacuum deposition of the desired metal onto cleaned glass substrates. Through careful control of the deposition-substrate temperature, deposition rate, and film thickness, Au islands were produced with specific surface plasmon resonance wavelengths. Specific detail of their production can be found in the literature. [14]

Silver nano-shells were produced via the method described by Mayer *et al* [15]. According to their report, a smooth shell of silver around latex spheres was accomplished by a two step reduction method that involved the use of NaH_2PO_2. The composite material was produced on 130, 220, 510, 720, and 860 nm diameter carboxylated latex spheres (diluted 1 drop in 10 mL of DDI water). A solution of sodium hypophosphite [70mM], latex spheres, and Ag {13 mM $AgNO_3$] soaked ~12 hours and were then reduced with NH_3 [4 % by vol., prepared fresh], and formaldehyde. Once the reaction is complete, the product has a semi-hydrophobic character and usually settles to the bottom of the reaction container. However, performing an extraction with hexane caused the particles to order along the interface between the two liquids. A simple Langmuir-Blodgett type film was produced by dipping a piece of microscope coverslip down through this interface and back out. The result was allowed to dry under nitrogen for 12-24 hours. Aqueous and ethanolic aliquots placed on the film did not disturb the adhesion of the resultant film to the coverslip. However, the end result was unsatisfactory with respect to our goals, and a new method was created where Ag was

added gradually in 300 μL aliquots to the reaction mixture, and the initial concentration of silver nitrate was also reduced to 2.5 mM. Figure 4 shows the evolution or tuning of the UV-vis extinction spectra obtained for Ag-latex composites formed from 630, 720, and 1000 nm cores as more silver was gradually reduced in solution. Some of these solutions were later immobilized on (3-aminopropyl)trimethoxysilane derivatized glass cover slips via the method described by Olson *et al*. [16]

3. RESULTS AND DISCUSSION

Figure 3 shows the SEM image of the Ag-latex composite films or nano-shells that were produced via the method described above. As can be seen the immobilized silver was irregular with respect to surface architecture and coverage of the latex spheres, but these films did produce SERS enhancements for pyridine and bacillus spores. The latter of these spectra are shown in figure 5. However, it is our goal to investigate rigidly defined surface structures in order to negate surface "hot spots" and nonlinear behavior. The initial solutions of nano-shells produced by gradual addition of AgNO3 showed promising surface plasmon absorption, but attempts at immobilizing these composites proved unsuccessful. Furthermore, attempts to reproduce the initial results failed. After immobilization, the nano-shell substrates of figure 4 exhibited an extinction spectrum of one band centered near 425 nanometers, characteristic of silver colloids. It should be noted, that at the time of this writing, there is no specific evidence that silver was immobilized on these latex spheres. However, each trial resulted in distinct differences between the solutions with varying size of latex spheres. A control solution with the same reactants and concentrations but excluded the latex spheres was used as a point of reference. The control solution exhibited visual and spectroscopic differences from each attempt to reproduce the initial nano-shells (*i.e.* it was much more turbid and the extinction spectra consisted of one band centered near 425 nm), but remained constant with respect to the previous control solutions. This data suggests that the latex spheres and their size play some role in the formation, or conjugation of the colloids, but we have yet to investigate this aspect.

Figure 6 shows the SERS spectra of vegetative *E. coli* again on both the vacuum FONS and Au-island film. The Au-island films showed no enhancement for the bacteria while the FONS spectrum could reproducibly show some amount of enhancement. It should be noted however that the entire surface of the FONS did not enhance the signal from the bacteria. Meanwhile, the Au-island film showed nearly identical enhancement between different points along the substrate. In fact, the signal remained constant even after rinsing the substrate with acetone and reapplying a sample at a randomly chosen point. This is shown in figure 7.

Similar reproducibility in spectra was found for the amine wet-FONS substrates. Figure 8 shows SER spectra of a stock solution of Rhodamine 6G spotted on an amine wet-FONS substrate. The more intense spectra is the result of a fresh layer of silver being deposited on top of the same amount of gold present for the other two substrates. The thickness of the metal coatings has yet to be determined, but each substrate was left in the reducing solution for the same amount of time. Figure 8 shows good reproducibility between two replicate substrates and that a quick fresh deposition of silver will result in higher enhancement as well. The signal to noise ratio (**S/N,** taken as the height of the 1504 cm-1 band above the zero baseline over the standard deviation of a flat portion of the spectra at the zero baseline—in each case the peak and the noise segments used were integrated over the same data points) of the two gold substrates was nearly identical at,16.086 and 15.898. The spectrum from the silver substrate exhibited a S/N of 50.585. The added enhancement of silver was used to investigate *bacillus stearothermophilus* ATCC 12980 spores that had been immobilized on the aminated polystyrene mask prior to the addition of gold colloid. The results are displayed in figure 9, but absent is the reproducibility seen previously with the Rhodamine 6G data in figure 8. The spores are stored as a suspension of 1 x 10^6 cfu/mL in a 60% ethanolic solution. The solution was placed on the dry latex sphere mask as a 100 μL drop, which spread over the substrate without aid and was allowed to dry in an ambient environment. It remains unclear whether the difference in response over different points on the substrate is the result of uneven coverage of the spores, or irregular surface architecture. SER spectra of spores were only achieved if the spores were immobilized first and gold/silver were subsequently deposited next to or directly on them (as SEM images have not been obtained the latter possibility cannot be ruled out). Wet-FONS substrates performed similarly to the amine wet-FONS as can be seen in figure 10 (S/N = 28.046), but attempts to investigate spores or large particles such as dilute suspensions of latex spheres failed, *i.e.* no enhanced signal was observed. Furthermore, these substrates showed poor reproducibility when compared to one another. It is our opinion that the method of initial immobilization on these substrates is quite random, which translates into uneven coverage in the subsequent electrodeless deposition of gold. It is our desire to use the bare latex sphere masks in conjunction with vapor deposition of gold under a vacuum.

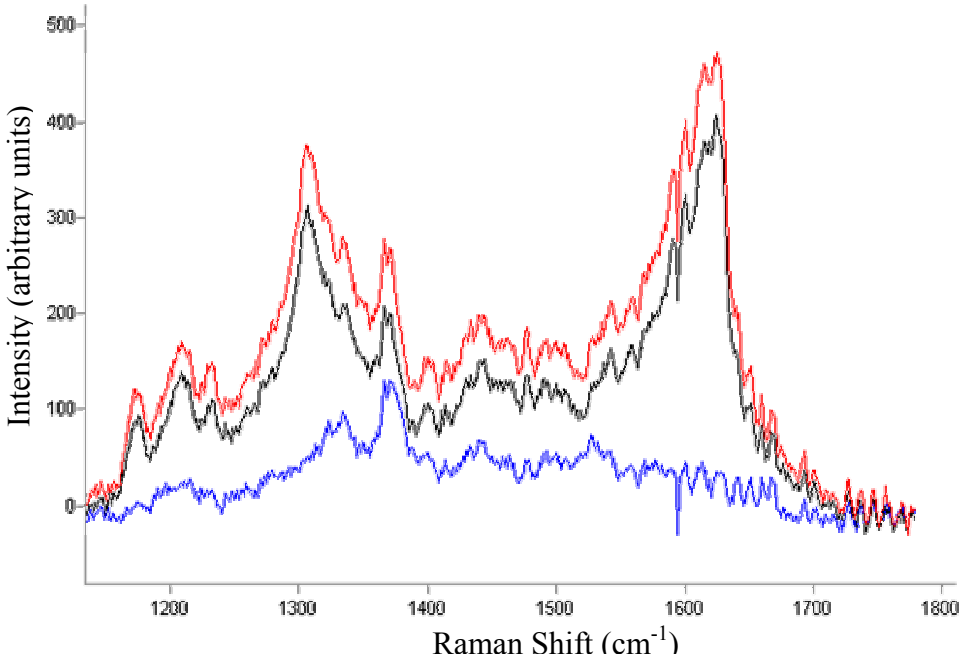

Figure 6: Spectra of vegetative E. coli over FONS (top two traces) and Zyvex Au-island film (bottom trace) which showed no enhancement

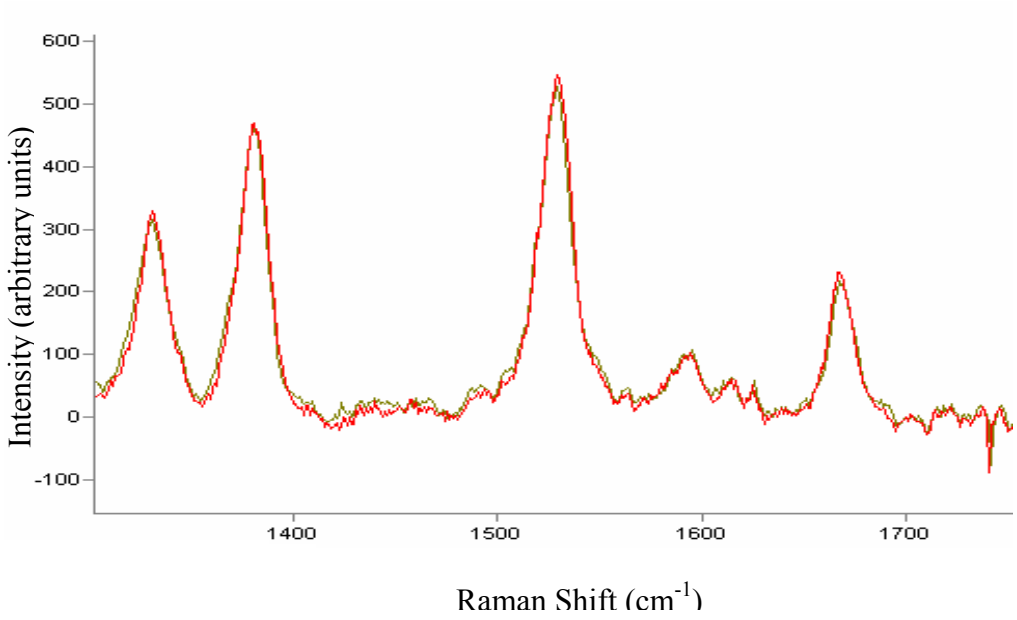

Figure 7: Spectra of Rhodamine 6G over the same Au-island substrate—the substrate was rinsed and re-coated between measurements

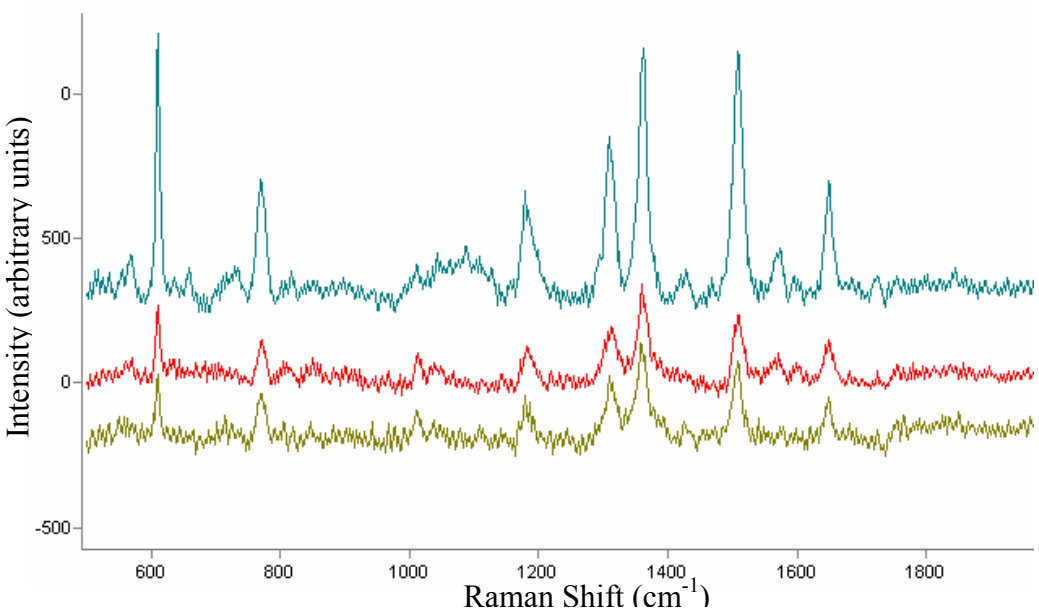

Figure 8: Spectra of Rhodamine 6G on an amine wet-FONS—offset for clarity, the top trace had a fresh layer of Ag deposited on the Au already present, the bottom two traces show two different Au only amine wet FONS substrates

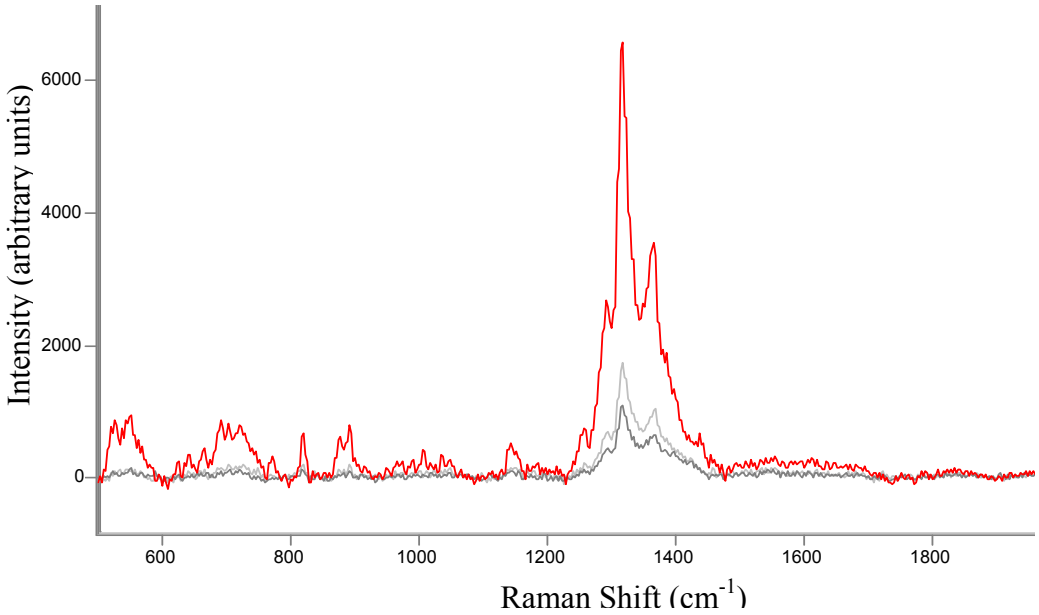

Figure 9: Spectra of *bacillus stearothermophilus* immobilized on a silver amine-wet FONS substrate—each spectrum is a different point along the substrate surface.

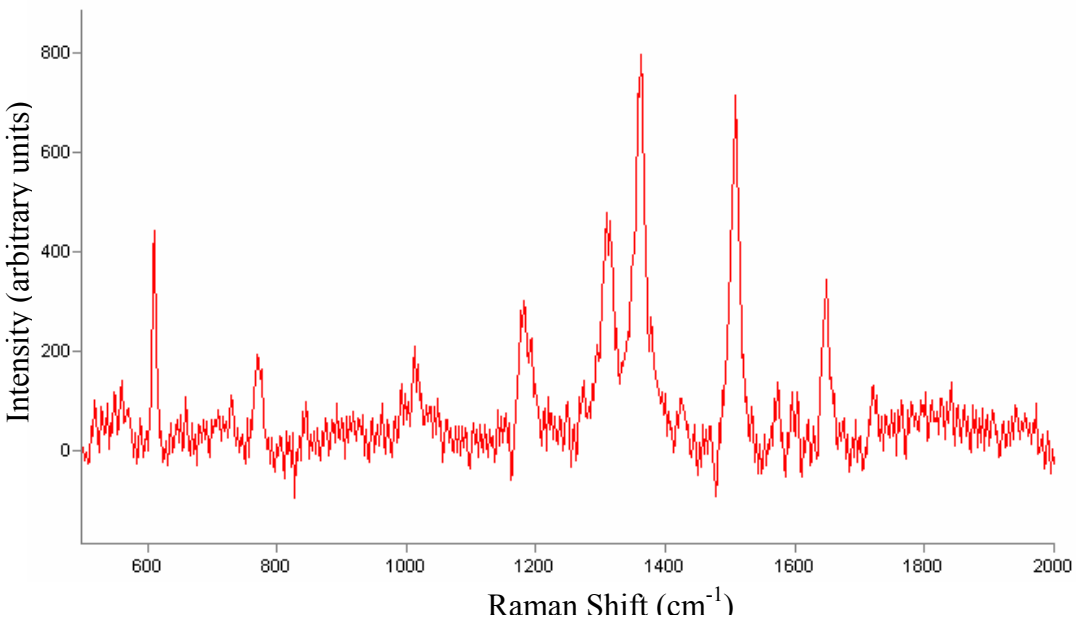

Figure 10: SER spectrum of Rhodamine 6G over wet-FONS substrate

4. CONCLUSIONS

These results are preliminary, and investigations are ongoing. SERS spectra of vegetative *E. coli* over a vacuum FONS, and *bacillus stearothermophilus* spores immobilized on amine wet-FONS substrates have been demonstrated. To date these substrates have shown uniform enhancement over large surface areas for chemicals, but these substrates showed no reproducibility when investigating large biological particles such as spores, or even uniform large particles such as polystrene latex spheres. We are attempting to incorporate the technique of optical trapping with NSL and electon-beam lithography substrates in order to force a single spore onto various, similarly tuned substrates to judge the effect of specific architecture relative to surface enhancement. Subsequently, this data will be compared to bulk sampling of spores with the instrumentation used in this report. As of yet, periodic particle arrays (**PPA**) have not been thoroughly investigated in our laboratory. Since these results are still quite preliminary, the PPA substrates may prove advantageous with further development, especially with derivatized staging substrates or thin surface coatings. Indeed, Riboh *et al* have reported improved regularity and adhesion for PPA production by the addition of surfactant to the latex sphere suspension used to prepare the staging mask. [17]

ACKNOWLEDGEMENTS

The Authors would like to thank Dr. Brian Cullum at the University of Maryland Baltimore County for use of his vacuum deposition chamber, and Dr Phil Boyd at ARL for the SEM images. Dr. Wayne Wiemer from Zyvex Inc. is also thanked for supplying two lots of Au-island films.

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